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CONTRASTING PATTERNS OF DIVERSITY AND POPULATION DIFFERENTIATION AT THE INNATE IMMUNITY GENE TOLL-LIKE RECEPTOR 2 (TLR2) IN TWO SYMPATRIC RODENT SPECIES

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Comparing patterns of diversity and divergence between populations at immune genes and neutral markers can give insights into the nature and geographic scale of parasite-mediated selection. To date, studies investigating such patterns of selection in vertebrates have primarily focused on the acquired branch of the immune system, whereas it remains largely unknown how parasite-mediated selection shapes innate immune genes both within and across vertebrate populations. Here, we present a study on the diversity and population differentiation at the innate immune gene Toll-like receptor 2 (TLR2) across nine populations of yellow-necked mice (*Apodemus flavicollis*) and bank voles (*Myodes glareolus*) in southern Sweden. In yellow-necked mice, TLR2 diversity was very low, as was TLR2 population differentiation compared to neutral loci. In contrast, several TLR2 haplotypes co-occurred at intermediate frequencies within and across bank vole populations, and pronounced isolation by distance between populations was observed. The diversity and differentiation at neutral loci was similar in the two species. These results indicate that parasite-mediated selection has been acting in dramatically different ways on a given immune gene in ecologically similar and sympatric species. Furthermore, the finding of TLR2 population differentiation at a small geographical scale in bank voles highlights that vertebrate innate immune defence may be evolutionarily more dynamic than has previously been appreciated.

Running title: TLR2 diversity and divergence in the wild

47 Keywords: coevolution, population differentiation, host-parasite interactions,
48 immunogenetics, innate immune defence, local adaptation, parasite-mediated
49 selection
50

Introduction

Depending on the specific type of host-parasite interaction, parasite-mediated selection can have radically different effects on the diversity and allele frequency distribution at host immune genes within populations. For example, 'arms race' coevolution between hosts and parasites can result in positive directional selection on host defence genes, and rapidly drive advantageous mutations to fixation (Bergelson et al. 2001; Woolhouse et al. 2002). Alternatively, balancing selection can act on immune defence genes as a result of negative frequency-dependent selection (Takahata and Nei 1990; Woolhouse et al. 2002) or heterozygote advantage (Doherty and Zinkernagel 1975; Apanius et al. 1997), and promote the maintenance of several alleles at intermediate frequencies.

Superimposed on these different types of parasite-mediated selection, spatial variation in the composition of the parasite assemblage will influence the scale at which these effects occur (Hedrick 2002). If the parasite community is homogenous across a host species' range, patterns of selection on host immune genes are expected to be similar across populations. If, however, the parasite community encountered by hosts varies in space, we expect diversifying selection by parasites across host populations. Thus, depending on the specific type of parasite-mediated selection, and the scale at which it occurs, patterns of allele frequency distribution at host defence genes will deviate from neutral expectations in characteristic ways. Population genetic analyses of immune genes can therefore give insights into the nature of parasite-mediated selection and elucidate the role parasites play in creating

and maintaining diversity within and across host populations (Haldane 1949; Thompson 1999; Buckling and Rainey 2002; Tiffin and Moeller 2006).

In vertebrates, the immune system consists of two branches, innate and acquired immunity (Klein 1993). Thus far, studies investigating spatial patterns of selection on immune genes in vertebrates have primarily focused on the major histocompatibility complex (MHC), receptors that present antigens to T cells and thereby stimulate acquired immune responses (Klein 1993). These genes typically show strong evidence of balancing selection (reviewed in Bernatchez and Landry 2003; Garrigan and Hedrick 2003; Piertney and Oliver 2006), but the role of spatially varying selection is mixed. Some studies have found evidence for parasite-driven population differentiation at MHC genes (Landry and Bernatchez 2001; Ekblom et al. 2007), while others have found that MHC allele frequency distributions are more homogenous across populations than expected under neutral processes (Piertney 2003; Sommer 2003).

In contrast, information on the type and / or spatial scale of parasite-mediated selection acting on other components of the vertebrate immune system, and in particular on genes of the innate branch of the vertebrate immune system, remains scarce. In fact, even for plants and invertebrates little is known about spatial variation in selection pressures shaping the immune system across natural populations (but see e.g., Moeller and Tiffin 2008). This hampers further progress in our understanding of evolutionary processes in host-parasite systems and disease ecology in natural populations (Jepson et al. 1997; Acevedo-Whitehouse and Cunningham 2006).

Innate immunity relies on a number of different receptors (so called pattern-recognition-receptors or PRRs), which recognise conserved structures (so called pathogen-associated molecular patterns or PAMPs) of specific groups of microorganisms (Kimbrell and Beutler 2001; Janeway and Medzhitov 2002; Akira et al. 2006). One of the best-studied groups of PRRs are Toll-like receptors (TLRs) in vertebrates (Medzhitov 2001; Vasselon and Detmers 2002; Takeda et al. 2003), with closely related PRRs also found in invertebrates (Toll receptors; Lemaitre et al. (1996)) and plants (e.g. FLS2 or EFR; Gomez-Gomez and Boller (2000); Zipfel et al. (2006)). TLRs belong to a multigene family, which has evolved by gene duplication (Zhou et al. 2007). Most mammals have 10 to 12 different TLRs, each recognising different pathogen structures (Roach et al. 2005). TLR2, for example, targets lipoproteins from cell walls of bacteria, whereas TLR3 recognises double-stranded RNA of viruses (Garantziotis et al. 2008). After stimulation with their respective ligands, TLRs initiate an intracellular signalling cascade that results in an inflammatory response in the infected tissue, as well as the stimulation of responses of the acquired immune defence (Medzhitov 2001; Akira and Takeda 2004). Because of their role in pathogen recognition, Toll-like receptors, like MHC molecules, have been suggested to be primary targets of parasite-mediated selection (Hughes and Friedman 2008). Yet, because TLRs recognise conserved molecular motifs in pathogens (PAMPs), their evolution is often believed to be constrained (Medzhitov and Janeway 1997). In line with this latter hypothesis, the few population genetic studies on TLRs performed to date (all in humans) found little evidence for population

differentiation, even when comparing populations across continents (Ferrer-Admetlla et al. 2008; Wlasiuk et al. 2009; Wlasiuk and Nachman 2010).

To improve our understanding of the direction and spatial scale of selection acting on innate immune genes in general, and TLRs in particular, we here present a study of patterns of TLR2 diversity and population differentiation in two common rodent species living in sympatry, the bank vole (*Myodes glareolus*) and the yellow-necked mouse (*Apodemus flavicollis*), across nine locations in southern Sweden. Because TLR2 genetic differentiation between populations could result from neutral processes or natural selection, we compared patterns of differentiation at TLR2 with those at neutral microsatellite loci to distinguish between the two processes. Importantly, by comparing two rodent species, we were able to investigate if parasite-mediated selection acts in similar ways on orthologous immune genes in ecologically similar host species living in sympatry.

Methods

Study species

The yellow-necked mouse (*Apodemus flavicollis)* is slightly larger than the bank vole (*Myodes glareolus)* (adult weight in our study population: 22 – 56 g and 15 – 40 g, respectively). Both species occur in central and eastern Europe, Scandinavia, the British Isles, and western Russia, but the range of the bank vole extends further north and east than that of the yellow-necked mouse (Corbet and Harris 1991). The bank vole prefers deciduous woodlands but also occurs in more open grassland, while the yellow-necked mouse

149 primarily occurs in deciduous woods, although the overlap in habitat use
150 between the two species is large. They also seem to share parasites to a
151 large extent. For example, at our study sites, the prevalence of the tick- and
152 flea-transmitted bacteria *Borrelia afzelii, Candidatus* Neoehrlichia mikurensis
153 and *Bartonella sp.* are similar in the two species (Hellgren et al. 2010; M.
154 Andersson & L. Råberg 2011). Population densities of the bank vole are
155 typically larger (3 – 5 times at our study sites; L. Råberg, unpublished data)
156 than those of the yellow-necked mouse. All our study sites are located in
157 southern Sweden. The bank vole colonized this area from the south shortly
158 after the last glaciation (around 11000 – 10000 years BP; Jaarola et al. 1999),
159 and given its current distribution, the yellow-necked mouse is likely to have a
160 similar colonization history (Jaarola et al. 1999).
161
162 *Samples*
163 Tissue samples from bank voles (N = 180) and yellow-necked mice (N = 127)
164 were obtained in 2008 at nine sites (henceforth termed 'populations') in
165 southern Sweden (Figure 1, Table 1) using live-traps (Ugglan Special No1,
166 Grahnab, Gnosjö, Sweden). The distance between sampled sites ranged from
167 0.3 to 342 km (Table S1).
168
169 *Toll-like receptor 2 (TLR2) sequencing*
170 The entire TLR2 coding region in bank vole and yellow-necked mouse is 2352
171 bp and 2355 bp, respectively (Tschirren et al. 2011). For this study, we
172 analysed a 1173 bp long fragment of TLR2 from bp 691 to 1863 in both
173 species. This amplicon contains most of the functionally relevant sites

174 involved in pathogen-recognition and TLR2-TLR1 heterodimerisation (Gautam
175 et al. 2006; Jin et al. 2007), and we previously showed that positive selection
176 has shaped codons within this region during the evolutionary history of
177 rodents (Tschirren et al. 2011).

178 Bank vole-specific primers (MglTLR2F: CATCCATCACCTGACCCTTC
179 and MglTLR2R: CCAGTAGGAATCCTGCTCG) were designed using the bank
180 vole TLR2 sequence (GenBank accession number HM215589) and the
181 program Primer3 (Rozen and Skaletsky 2000). Yellow-necked mouse-specific
182 primers (AflTLR2F: TCACCTGACGCTTCACTTGAG and AflTLR2R:
183 CATGAGGTTCTCCACCCAGT) were designed using the yellow-necked
184 mouse TLR2 sequence (GenBank accession number HM215601) and the
185 same program. There was no indication that we amplified more than one
186 locus with these primers (i.e. clean sequences), and a NCBI GenBank blast of
187 the obtained sequences revealed similarities with TLR2 of other, even
188 phylogentically distant species, but not with other TLRs.

189 Total genomic DNA was extracted from the biopsies following the
190 protocol of Laird et al. (1991). PCR reactions were performed in a total
191 volume of 25 µl including 25 ng of total genomic DNA, 0.125 mM of each
192 dNTP, 2.0 mM $MgCl_2$, 1x PCR Buffer (Applied Biosystems, Foster City, CA,
193 USA), 1 mM of each primer, and 2.5 U AmpliTaq DNA polymerase (Applied
194 Biosystems, Foster City, CA, USA) on a GeneAmp PCR Systems 9700
195 thermocycler (Applied Biosystems, Foster City, CA, USA). The PCR protocol
196 included an initial denaturation step at 94 °C for 5 minutes, followed by 37
197 cycles of denaturation at 94 °C for 30 seconds, annealing at 57 °C for 30
198 seconds and extension at 72 °C for 150 seconds. The program ended with a

199 final extension step at 72 ℃ for 10 minutes. The PCR products were
200 sequenced in both directions on an ABI Prism 3730 capillary sequencer
201 (Applied Biosystems, Foster City, CA, USA) using Big Dye terminator v3.1
202 chemistry (Applied Biosystems, Foster City, CA, USA). Sequences were
203 processed, assembled and aligned using the program Geneious 5.0.4.
204 (Drummond et al. 2009) and all polymorphisms were examined by eye. TLR2
205 haplotypes were reconstructed with the program PHASE 2.1. (Stephens et al.
206 2001; Stephens and Scheet 2005) using default settings. Haplotypes could be
207 inferred with very high probabilities in both species (see Table S2 for
208 confidence probabilities of phase calls). If a haplotype was observed only
209 once in the dataset, we repeated the PCR and sequencing reactions to
210 confirm the sequence and avoid overestimation of diversity due to
211 amplification or sequencing errors. Haplotype data (considering synonymous
212 and nonsynonymous substitutions in TLR2) were used in the statistical
213 analyses. To compare differences in haplotype frequencies between species,
214 we considered only nonsynonymous differences between haplotypes.
215 Haplotypes were submitted to NCBI GenBank.
216
217 *Microsatellite analysis*
218 In order to test whether TLR2 alleles behaved differently from neutral
219 expectations, we genotyped the bank vole and yellow-necked mouse samples
220 at putatively neutral microsatellite loci. For bank voles we used the following
221 eight markers: Cg16E2, Cg13F9, Cg7E5, Cg3A8, Cg17A7, Cg12A7, Cg3A6,
222 and Cg4F9 (Rikalainen et al. 2008). Amplifications were performed in a 10 µl
223 volume using Qiagen Multiplex Kit (Qiagen AB, Sollentuna, Sweden) with

fluorescent-labelled forward primers and non-labelled reverse primers (0.2 μM each) on a GeneAmp PCR Systems 9700 thermocycler (Applied Biosystems, Foster City, CA, USA). PCR conditions for the first multiplex set, including markers Cg16E2, Cg13F9, Cg7E5, Cg3A8, and Cg17A7, consisted of an initial denaturation step at 95 °C for 15 min, followed by 6 cycles of 30 sec at 94 °C, 90 sec at 56 °C minus 1 °C per cycle, 60 sec at 72 °C, and 27 cycles of 30 sec at 94 °C, 90 sec at 51 °C, 60 sec at 72 °C followed by a final extension step of 15 min at 70 °C. PCR conditions for the second multiplex set, including markers Cg12A7, Cg3A6, and Cg4F9, consisted of an initial denaturation step at 95 °C for 15 min, followed by 3 cycles of 30 sec at 94 °C, 90 sec at 55 °C minus 1 °C per cycle, 60 sec at 72 °C, and 30 cycles of 30 sec at 94 °C, 90 sec at 53 °C, 60 sec at 72 °C followed by a final extension step of 15 min at 70 °C.

Yellow-necked mouse samples were analysed at the following five microsattelite loci: GTTC4A, GTTD9A (Makova et al. 1998), MSAf-16 (Gockel et al. 1997), As-20 (Harr et al. 2000), and MSAA-5 (Ohnishi et al. 1998). PCR conditions for the multiplex set including all five markers consisted of an initial denaturation step at 95 °C for 15 min, followed by 30 cycles of 30 sec at 94 °C, 90 sec at 57 °C, 60 sec at 72 °C followed by a final extension step of 15 min at 68 °C.

PCR fragments were separated by capillary electrophoresis on an ABI 3730XL sequencer (Applied Biosystems, Foster City, CA, USA). Fragment length was determined in comparison to an internal size standard (LIZ 500) using the program ABI Prism GeneMapper 3.0 (Applied Biosystems, Foster City, CA, USA). The potential occurrence of null alleles was assessed using

the software MicroChecker 2.2.3. (Van Oosterhout et al. 2004). In bank voles, Cg16E2 showed an excess of homozygotes in two populations (IB and MD), and Cg7E5 and Cg3A8 showed an excess of homozygotes in one population each (HE and KN, respectively). In yellow-necked mice, MSAf-16 (IB), As-20 (IB) and GTTD9A (KS) showed an excess of homozygotes in one population, each. Because the number of observed significant tests (7) is close to what is expected by chance (5.9) given the number of test performed (117), and because there were no systematic patterns of null allele occurrence across markers or populations, we included all microsatellite loci in the statistical analyses.

To determine whether the microsatellite loci were indeed neutral (i.e. not linked to genes under selection), we performed an outlier analysis in Arlequin 3.5.1.2. (Excoffier and Lischer 2010). We performed coalescent simulations using a finite island model to obtain the neutral distribution of $F_{ST.}$ For each species, we generated 50000 paired values of F_{ST} and heterozygosity, which were used to calculate the 0.975 and 0.025 quantiles of the neutral distribution (Beaumont and Nichols 1996). We treated the sampled sites as one group and set the number of demes as the number of sampled sites. All microsatellite loci fell inside the 95% confidence interval (Figure S1), indicating that they are indeed neutral.

Statistical analyses

Within population diversity

To estimate genetic diversity at TLR2 and microsatellite loci within populations we calculated for each species the number of haplotypes (*h*), the number of

haplotypes when considering only nonsynonymous substitutions (h_{nons}), the mean number of microsatellite alleles ($K_{average}$) and allelic range (K_{range}), gene diversity or expected heterozygosity (H_d *or* H_E), observed heterozygosity (H_O), nucleotide diversity (π), the number of nonsynonymous substitutions per nonsynonymous site (π_a) and the number of synonymous substitutions per synonymous site (π_s) using the programs Arlequin 3.5.1.2. (Excoffier and Lischer 2010) or DNAsp 5.10.01 (Librado and Rozas 2009). Linkage disequilibrium between pairs of loci and deviations from Hardy–Weinberg equilibrium for each locus were tested in Arlequin 3.5.1.2. (Excoffier and Lischer 2010). For all statistical analyses, sequential Bonferroni corrections for multiple testing were applied whenever applicable (Holm 1979).

Between population differentiation

Comparing differentiation at neutral microsatellite loci and protein coding loci (like TLR2) is complicated by the fact that microsatellite loci typically have much higher mutation rates. In addition to traditional F_{ST} estimates of population differentiation, we therefore also calculated G'_{ST} (Hedrick 2005) and Jost's D (Jost 2008) (which both take mutation rates into account) as recommended by Meirmans and Hedrick (2011). It should be noted, however, that F_{ST}, G'_{ST}, and Jost's D measure differentiation in fundamentally different ways, and that the connection of G'_{ST} and D to evolutionary theory is not yet clear (Whitlock 2011). We calculated pairwise F_{ST} (Weir and Cockerham 1984) for each species in Arlequin 3.5.1.2. (Excoffier and Lischer 2010). In addition, we calculated standardized levels of population divergence corrected for the maximum level of population divergence attainable G'_{ST} (Hedrick

2005). This measure takes into account that microsatellite loci and TLR2 are likely to differ in their levels of heterozygosity, and thus in the maximum level of population divergence attainable (Hedrick 2005). We used the genetic differentiation software described in Neff and Fraser (2010) to calculate G'_{ST}. 95% confidence intervals for G'_{ST} were estimated by resampling individuals (TLR2) or both individuals and loci (microsatellites) with replacement 1000 times (Neff and Fraser 2010). This allowed us to compare TLR2 G'_{ST} confidence intervals relative to the microsatellite G'_{ST} confidence intervals between pairs of sites. Finally, we calculated Jost's *D* (Jost 2008), which evaluates population differentiation by quantifying genetic diversity within populations based on the effective number of alleles using the program SMOGD 1.2.5. (Crawford 2010) with 1000 bootstrap replicates.

Isolation by distance

Patterns of isolation by distance (IBD) were assessed within each species by testing the correlation between genetic distance (F_{ST} / (1 – F_{ST})) and geographical distance (log km, Table S1) for both TLR2 and microsatellite loci in Arlequin 3.5.1.2. (Excoffier and Lischer 2010). Geographical distances between sites were calculated from their latitude and longitude using an online geographical distance calculator. Statistical significance was evaluated using Mantel tests with 10000 permutations. We estimated the strength and direction of the correlation (*r*) and the coefficient of determination (R^2) between genetic and geographical distance, as well as the slope of the regression (*b*) of genetic distance against geographical distance. Using the MantelPiece *R*-script version 1.0 (available at

http://www.erikpostma.net/resources.html), we then tested if the differences in the slopes of the regression (Δb) between genetic differentiation based on TLR2 and geographical distance, and genetic differentiation based on microsatellite loci and geographical distance deviated significantly from zero. MantelPiece uses an extension of the standard Mantel test, and compares the observed Δb to the distribution of Δb after randomising the geographical distance matrix 30000 times.

Additionally, we performed partial Mantel tests where we correlated the genetic distance based on TLR2 (F_{ST} / (1 – F_{ST})) to geographical distance (log km) while keeping differentiation at microsatellite loci constant using Arlequin 3.5.1.2. (Excoffier and Lischer 2010). Statistical significance was evaluated using 10000 permutations. This test provides evidence for a correlation between geographical distance and TLR2 differentiation that is independent of demographic or stochastic processes (Ekblom et al. 2007).

Results

Within population diversity

We observed a high TLR2 diversity in bank voles whereas TLR2 diversity was very low in yellow-necked mice (Table 2). We found 37 polymorphic sites in TLR2, including 17 non-synonymous substitutions, in the bank vole data set, whereas only six polymorphic sites, including two non-synonymous substitutions, were found in yellow-necked mice. Nucleotide diversity (π) in TLR2 was on average 27 times higher and gene diversity (H_d) was on average seven times higher in bank voles than in yellow-necked mice (Table 2). All yellow-necked mouse populations showed a negative, but non-

significant Tajima's *D* for TLR2 (overall Tajima's *D* = -1.54, 0.10 > *P* > 0.05). In bank voles, Tajima's *D* was mostly positive (overall Tajima's *D* = 0.317, *P* > 0.05). However, values differed widely across populations. For example, population Häglinge (HA) showed a significantly positive Tajima's *D* (*D* = 2.19, *P* < 0.05), whereas population Herseby (HE) showed a significantly negative Tajima's *D* (*D* = -2.07, *P* < 0.05) (Figure 2).

The number and frequency of TLR2 haplotypes (h_{nons}) differed markedly between the two species. In bank voles, we observed three common haplotypes (frequencies > 15% each), which were found at eight or nine sampling sites (out of nine sampling sites in total) (Table 2, Figure 1). Two additional haplotypes occurred at frequencies of 1.4% and 2.2%, and were found at four sites each (Table 2, Figure 1). Another nine bank vole haplotypes with frequencies < 1.2% were found at one or two sites (Table 2, Figure 1). In yellow-necked mice, one TLR2 haplotype with an overall frequency of 94.5% was predominant at all nine sites (Table 2, Figure 1). Two other haplotypes were observed at three and seven sites, with overall frequencies of 4% and 1.5%, respectively.

These marked differences in TLR2 diversity between bank vole and yellow-necked mouse were not reflected at neutral microsatellite loci, where levels of diversity and heterozygosity were similar in the two species (Table 2).

No indication for linkage disequilibrium between pairs of microsatellite loci or deviation from Hardy-Weinberg equilibrium within loci was found (all *P* > 0.05 after Bonferroni correction).

Between population differentiation

We observed significant population differentiation (F_{ST}) based on microsatellite loci in bank voles even at a small spatial scale (i.e. within the Revinge area), with only one pairwise comparison (out of 36) not reaching statistical significance after Bonferroni correction (Table 3). Differentiation between bank vole populations (F_{ST}) based on TLR2 was very high in pairwise comparisons that involved the sites Hemmeströ, Istaby and Herseby, but was lower within the Revinge area (Table 3).

Yellow-necked mouse population differentiation (F_{ST}) based on microsatellite loci reached statistical significance in 20 of 36 pairwise comparisons after Bonferroni correction (Table 3). Differentiation (F_{ST}) at TLR2, however, was very low and non-significant in all pair wise comparisons of yellow-necked mouse populations.

To evaluate if populations are more or less differentiated at TLR2 than expected under neutral processes (e.g., founder effects, drift or migration), we compared the 95% confidence intervals of the standardized levels of population divergence (G'_{ST}) based on TLR2 with the 95% confidence intervals of the standardized levels of population divergence (G'_{ST}) based on microsatellite loci (Figure 3). No clear pattern was observed across bank vole populations. In most pair wise comparisons, population differentiation based on TLR2 was similar to what was observed at neutral loci. However, statistically significant higher or lower population differentiation at TLR2 was observed in four pairwise comparisons (Figure 3). In the yellow-necked

mouse, population differentiation based on TLR2 was generally lower than differentiation at neutral loci (Figure 3).

In addition to F_{ST} and G'_{ST}, we calculated overall levels of population differentiation D (Jost 2008). This measure evaluates population differentiation by quantifying genetic diversity within populations based on the effective number of alleles, and is independent of the average within-population heterozygosity and mutation rate (Jost 2008). In bank voles, population differentiation at TLR2 (D_{est} = 0.186, 95% CI: 0.180 – 0.319) was somewhat higher than population differentiation at microsatellite loci (harmonic mean D_{est} = 0.165; see Table S3 for D_{est} and 95% CI for each microsatellite locus). In yellow-necked mouse, population differentiation at TLR2 (D_{est} = 0.001, 95% CI: 0.001 – 0.021) was considerably lower than population differentiation at microsatellite loci (harmonic mean D_{est} = 0.270; see Table S3 for D_{est} and 95% CI for each microsatellite locus).

Isolation by distance

In bank voles, we observed significant isolation by distance at TLR2 (Mantel test: r = 0.572, R^2 = 0.327, P = 0.002; Figure 4), whereas no significant isolation by distance was observed at neutral loci (Mantel test: r = 0.308, R^2 = 0.099, P = 0.163; Figure 4). The association between F_{ST} based on TLR2 and geographical distance was only slightly influenced by the concurrent pattern observed at neutral loci, because a partial Mantel test, in which we corrected for differentiation at microsatellite loci, provided comparable results (Partial Mantel test: r = 0.561, P = 0.009). The difference in the slopes (Δb) between TLR2 genetic distance and geographical distance, and between neutral

422 genetic distance and geographical distance was more extreme than expected
423 by chance (observed Δb: -0.108, Randomisation test: $P = 0.025$) indicating
424 that TLR2 and neutral markers show significantly different patterns of isolation
425 by distance.
426 In yellow-necked mice, the opposite pattern was observed. Here, we
427 observed significant isolation by distance at microsatellite loci (Mantel test: $r =$
428 0.650, $R^2 = 0.424$, $P = 0.0001$; Figure 4) whereas no significant isolation by
429 distance was observed at TLR2 (Mantel test: $r = -0.231$, $R^2 = 0.053$, $P =$
430 0.833; Figure 4). The association between F_{ST} based on TLR2 and
431 geographical distance became even more negative when controlling for the
432 concurrent pattern observed at neutral loci (Partial Mantel test: $r = -0.542$, $P =$
433 0.996). The difference in the slopes (Δb) between TLR2 genetic distance and
434 geographical distance, and between neutral genetic distance and
435 geographical distance was more extreme than expected by chance (observed
436 Δb: 0.0435, Randomisation test: $P = 0.002$), again indicating that TLR2 and
437 neutral markers show significantly different patterns of isolation by distance,
438 but that patterns are reversed compared to the bank vole.
439
440 Discussion
441 The patterns of diversity and population differentiation observed at the innate
442 immune gene TLR2 and at neutral markers indicate that selection has been
443 acting in strikingly different ways on TLR2 in two ecologically similar rodent
444 species living in sympatry. In the yellow-necked mouse, one TLR2 haplotype
445 was predominant at all studied sites with an overall allele frequency of 95%.
446 Also, differentiation at TLR2 between yellow-necked mouse populations was

much lower than expected under neutral processes, and no isolation by distance was observed. These patterns suggest that one TLR2 variant has been favoured in a selective sweep that has eliminated most of the variation at this immune receptor in yellow-necked mice. Furthermore, selection in favour of this single TLR2 variant appears to have been uniform across all studied yellow-necked mouse populations in southern Sweden.

In bank voles, a very different pattern emerged. Here, we observed a much higher diversity at TLR2, and found 14 haplotypes (h_{nons}) across populations, three of which occurred at frequencies of > 15% each. Furthermore, we observed population differentiation and pronounced isolation by distance for TLR2 across bank vole populations. These patterns of isolation by distance were still present when controlling for the concurrent pattern at neutral loci, demonstrating that TLR2 isolation by distance cannot be explained by stochastic or demographic processes alone. Together, these results indicate that selection on the bank vole TLR2 is spatially heterogeneous, and that differences in selective pressures become stronger with increasing geographical distance between populations. Such patterns of isolation by distance can arise if the composition of the pathogen community varies gradually in space and different TLR2 alleles are best at recognizing different parasite species or genotypes (that is, a host genotype x parasite species or host genotype x parasite genotype interaction for disease susceptibility). Alternatively, a particular allele might be best at recognizing all pathogens of a given class, but also carry a cost, for example by increasing the propensity to develop inflammatory disease (Anders et al. 2005; Papadimitraki et al. 2007). A common SNP (Asp299Gly) in the human TLR4,

for example, is known to increases susceptibility to a wide range of pathogens, but at the same time reduces the risk of inflammatory disease (Cook et al. 2004; Schröder and Schumann 2005). If there is spatial variation in overall pathogen abundance (e.g., a latitudinal cline), such trade-offs could promote population differentiation at host immune genes.

An alternative scenario is that the observed patterns of diversity and population differentiation in the bank vole are a result of neutral or slightly deleterious mutations in TLR2. Yet, several of our findings seem incompatible with such a scenario. For example, although neutral or slightly deleterious mutations could cause the high TLR2 diversity, we would not expect such mutations to lead to the pronounced isolation by distance (significantly stronger than at neutral loci) observed across bank vole populations.

The population differentiation at the bank vole TLR2 contrasts with patterns for human TLRs, where there is little evidence for population differentiation, even at a global scale (Ferrer-Admetlla et al. 2008; Wlasiuk and Nachman 2010). Our result thus indicates that the pathogenic environment may play an important role in shaping innate immune receptors, thereby creating and / or maintaining diversity in innate immunity in natural populations. As has been shown both theoretically and empirically, such differences in local selection pressures by parasites can lead to rapid population divergence and local adaptation (Thompson 1999; Schulte et al. 2010), and may ultimately facilitate speciation processes in hosts (Haldane 1949; Buckling and Rainey 2002).

What could cause the pronounced differences in TLR2 diversity and population differentiation between the two rodent species? Phylogenetic

497 analyses of long-term patterns of selection (across speciation events) on
498 TLR2 in rodents showed that at least some TLR2 codons have been subject
499 to positive selection (Tschirren et al 2011). The same pattern of positive
500 selection has been found in other TLRs in primates (Wlasiuk and Nachman
501 2010). In contrast, there is no evidence for long-term balancing selection on
502 TLRs (unlike in, for example, the MHC; e.g. Piertney and Oliver (2006)). Thus,
503 the long-term evolution of TLRs seems to be characterized by occasional
504 selective sweeps in at least some lineages. One explanation for the
505 contrasting patterns in bank voles and yellow-necked mice is that the two
506 species have been caught at different stages of this process. If selective
507 sweeps are generally initiated by the random occurrence of a new, adaptive
508 mutation in one species (rather than the emergence of a novel pathogen that
509 imposes selection on the standing genetic variation in several species),
510 sweeps will not be synchronized across species. When taking a genetic
511 snapshot at any one time, different species might thus be caught at different
512 stages of TLR2 evolution. In the present case, the yellow-necked mouse
513 would be at the end of a selective sweep, whereas the bank vole is in the
514 process of diversification and local adaptation (i.e. in between two sweeps).
515 Once a new globally adaptive mutation appears in the bank vole, the current
516 pattern of local adaptation will be swept away.

517 Alternatively, species-wide selective sweeps might be limited to some
518 lineages, while other lineages consistently experience spatially varying
519 selection. One could imagine several different reasons for such species-
520 specific patterns of selection. Different host species could, for example, be
521 affected by host-specific parasites, which differ in their spatial dynamics.

However, the common rodent pathogens that are recognised by TLR2 are generalists (e.g. *Borrelia afzelii* (Hellgren et al. 2010), *Staphylococcus sp*. (Hauschild et al. 2010), or *Mycobacterium microti* (Cavanagh et al. 2002)). Thus host-specific parasites seem unlikely to explain the contrasting patterns of TLR2 diversity and differentiation observed in bank voles and yellow-necked mice. If the composition of the parasite community is similar in the two species, then species-specific patterns of selection on TLR2 might be due to intrinsic differences between the species. First, constraints induced by other components of the immune system that interact directly with TLR2 (e.g., TLR1 or TLR6, which form heterodimers with TLR2; Jin et al. 2007) may influence TLR2 evolution in a species-specific way. Second, the relative importance of TLR2-dependent and TLR2-independent defence mechanisms might differ between the two species. The vertebrate immune system is highly redundant and if one of the host species primarily relies on TLR2-independent components (e.g., the complement system; Kurtenbach et al. 1994; Kurtenbach et al. 1998) to fight prevalent pathogens, this could affect patterns of selection on TLR2. Third, the two species might differ in their propensity to develop autoimmune disease, which will shape the balance between maintaining a strong enough pro-inflammatory response to fight pathogens and keeping inflammation at low enough levels to avoid autoimmune diseases (Anders et al. 2005; Papadimitraki et al. 2007). Differences in the optimal solution of this trade-offs could shape the strength and direction of selection acting on innate immune genes (Drexler and Foxwell 2010). Disentangling the relative importance of these different explanations for species-specific patterns of selection on immunity genes will require further studies of both

long- and short-term evolutionary dynamics of a wider range of immunity genes.

Conclusion

The different patterns of diversity and population differentiation at TLR2 in the two rodent species indicate that parasite-mediated selection may not only act in different ways across environments (e.g., Wegner et al. 2003; Dionne et al. 2007; Evans and Neff 2009), but also differently on relatively closely related species co-occurring in the same environment. These results highlight that it is difficult to extrapolate findings from one system to another. They also caution against making general conclusions about the type and direction of selection acting on a particular immune gene, as patterns of selection might be more diverse and context-dependent than has previously been appreciated. In particular, vertebrate innate immune genes have often been described as evolutionary static and not a primary target of parasite-mediated selection (e.g., Medzhitov and Janeway 1997; Mukherjee et al. 2009). As we show here, this view might be too simplistic, and it might hamper further progress in our understanding of host-parasite interactions and disease ecology in natural populations.

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579 Figure Legends

580

581 Figure 1. Allele frequencies at nine sampling sites in southern Sweden for
582 bank vole (A) and yellow-necked mouse (B). Shown are the five most
583 common haplotypes (based on nonsynnonymous changes only; h_{nons}) for
584 bank vole. These five haplotypes were observed at four sites or more, each.
585 The other nine haplotypes (frequency < 1.2% each) were collapsed into a
586 sixth category 'rare' (orange). Within this 'rare' category four alleles were
587 observed at two sites, each, and five alleles were found at one site, each. All
588 three nonsynonymous haplotypes are shown for yellow-necked mouse. Each
589 of these haplotypes was observed at three sites or more. Note that haplotypes
590 are not shared by the two species. KN, Kalvs mosse north; KS, Kalvs mosse
591 south; SI, Silvåkra; MD, Myrdungen; LL, Lavelund; HA, Häglinge; HS,
592 Hemmeströ; IB, Istaby; HE, Herseby.

593

594 Figure 2. Tajima's *D* for TLR2 in bank vole (filled circles) and yellow-necked
595 mouse (open circles) at nine sites in southern Sweden. A negative Tajima's D
596 is indicative of a population size expansion and / or a selective sweep and / or
597 purifying selection. A positive Tajima's D is indicative of a decrease in
598 population size and / or balancing selection acting on TLR2. KN, Kalvs mosse
599 north; KS, Kalvs mosse south; SI, Silvåkra; MD, Myrdungen; LL, Lavelund;
600 HA, Häglinge; HS, Hemmeströ; IB, Istaby; HE, Herseby.

601

602 Figure 3. Comparison of standardized levels of population divergence (G'_{ST})
603 based on microsatellite loci (open circles) and TLR2 (filled circles) for each

pairwise comparison between nine sites for bank vole (A) and yellow-necked mouse (B). Error bars indicate 95% confidence intervals as estimated by resampling of individuals (TLR2) or both individuals and loci (microsatellites) with replacement 1000 times (*$P < 0.05$). KN, Kalvs mosse north; KS, Kalvs mosse south; SI, Silvåkra; MD, Myrdungen; LL, Lavelund; HA, Häglinge; HS, Hemmeström; IB, Istaby; HE, Herseby.

Figure 4. Relationship between genetic distance (F_{ST} / (1- F_{ST})) and the logarithm of geographical distance (log km) across nine sampled sites for bank vole (A) and yellow-necked mouse (B). Genetic distances were calculated based on microsatellite loci (open circles, dashed line) and TLR2 (filled circles, solid line). Regression lines are shown.

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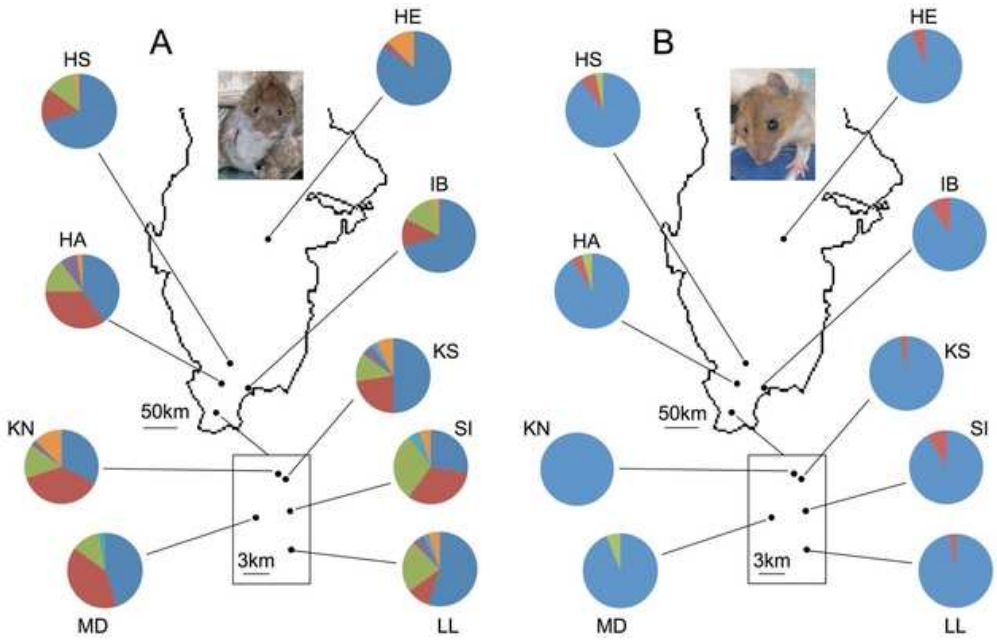


Figure 1 EPS format
53x33mm (300 x 300 DPI)

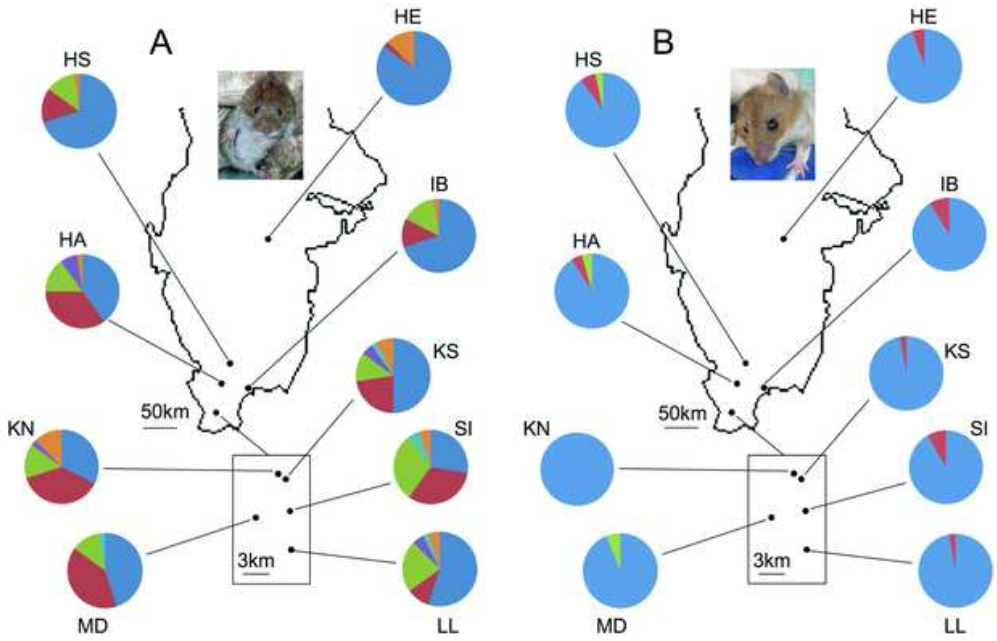
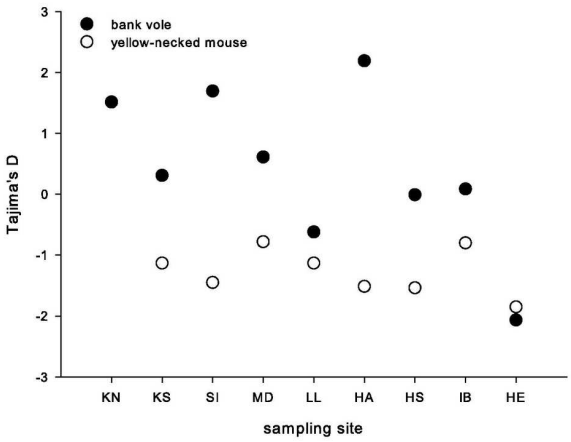
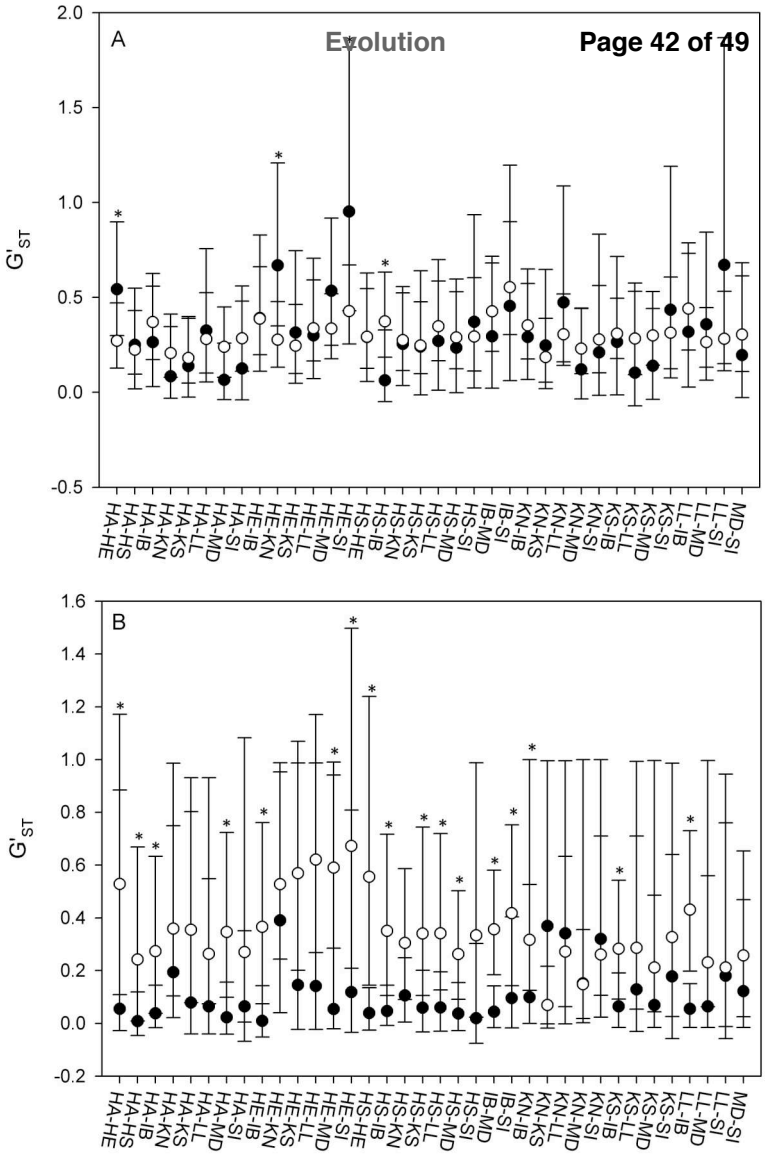
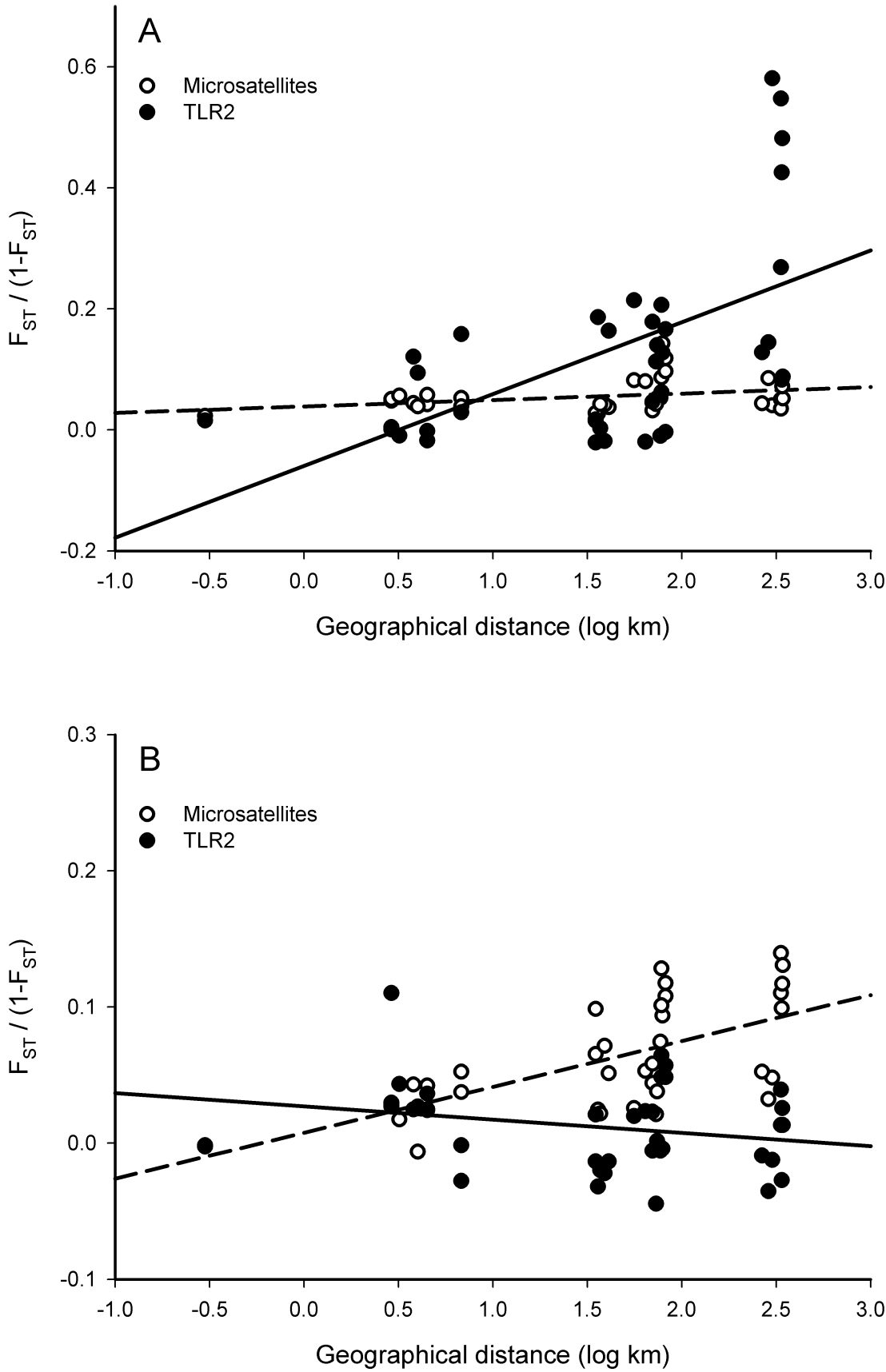


Figure 1 TIFF format
52x33mm (300 x 300 DPI)



117x165mm (300 x 300 DPI)


A
B
G'ST
HA-HE
HA-HS
HA-IB
HA-KN
HA-KS
HA-LL
HA-MD
HA-SI
HE-IB
HE-KN
HE-KS
HE-LL
HE-MD
HE-SI
HS-HE
HS-IB
HS-KN
HS-KS
HS-LL
HS-MD
HS-SI
IB-MD
IB-SI
KN-IB
KN-KS
KN-LL
KN-MD
KN-SI
KS-IB
KS-LL
KS-MD
KS-SI
LL-IB
LL-MD
LL-SI
MD-SI


A
Microsatellites
TLR2
$F_{ST} / (1-F_{ST})$
0.6
0.4
0.2
0.0
-0.2
-1.0
-0.5
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Geographical distance (log km)
B
Microsatellites
TLR2
$F_{ST} / (1-F_{ST})$
0.3
0.2
0.1
0.0
-0.1
-1.0
-0.5
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Geographical distance (log km)

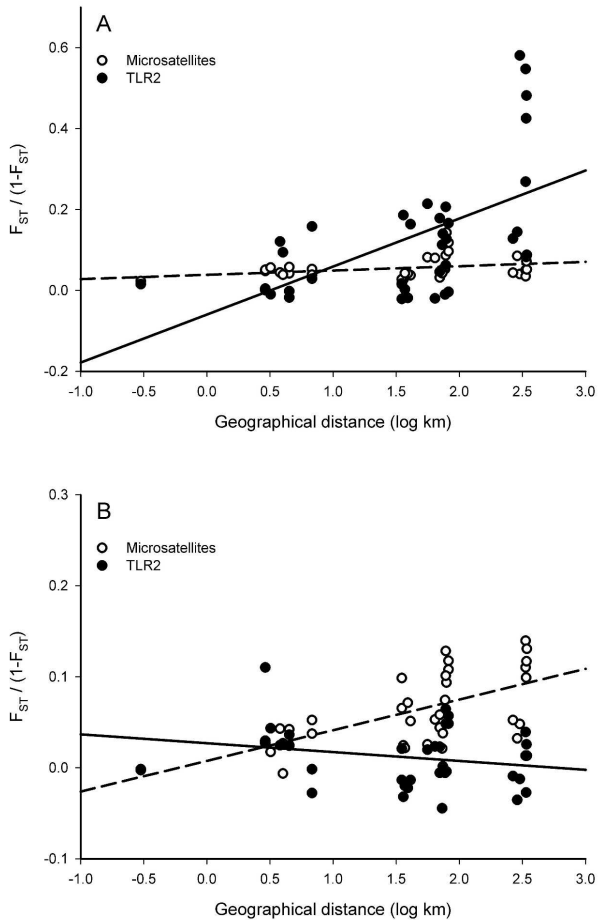


Figure 4 in EPS format
297x420mm (300 x 300 DPI)

Table 1. Sampling sites, coordinates of sites, site ID and number (*N*) of bank vole (BV) and yellow-necked mouse (YNM) samples.

Sampling site	Coordinates	Site ID	BV (*N*)	YNM (*N*)
Revinge - Kalvs mosse north	N 55° 42.470', E 13° 29.216'	KN	20	17
Revinge - Kalvs mosse south	N 55° 42.470', E 13° 29.216'	KS	20	18
Revinge - Silvåkra	N 55° 40.958', E 13° 29.806'	SI	20	6
Revinge - Myrdungen	N 55° 40.415', E 13° 26.918'	MD	20	16
Revinge - Lavelund	N 55° 38.822', E 13° 29.131'	LL	20	18
Häglinge	N 55° 59.625', E 13° 42.308'	HA	20	11
Hemmeströ	N 56° 19.200', E 13° 44.460'	HS	20	15
Istaby	N 56° 1.071', E 14° 36.522'	IB	20	17
Herseby	N 58° 35.002', E 15° 7.780'	HE	20	9

Table 2. TLR2 and microsatellite diversity at the nine sampled sites for bank vole (A) and yellow-necked mouse (B). Number of individuals, number of TLR2 haplotypes (*h*) or average number and range of microsatellite alleles (*K*); number of TLR2 haplotypes when considering only nonsynonymous substitutions (h_{nons}); gene diversity or expected heterozygosity (H_d / H_E); observed heterozygosity (H_O); nucleotide diversity (π); the number of nonsynonymous substitutions per nonsynonymous site (π_a); the number of synonymous substitutions per synonymous site (π_s). KN, Kalvs mosse north; KS, Kalvs mosse south; SI, Silvåkra; MD, Myrdungen; LL, Lavelund; HA, Häglinge; HS, Hemmeströ; IB, Istaby; HE, Herseby.

A.	all	KN	KS	SI	MD	LL	HA	HS	IB	HE
N	180	20	20	20	20	20	20	20	20	20
TLR2										
h	24	11	11	6	8	7	8	7	6	6
h_{nons}	14	7	8	5	4	6	5	4	4	4
H_d	0.807	0.842	0.841	0.755	0.789	0.803	0.808	0.664	0.745	0.667
H_O		0.800	0.800	0.850	0.750	0.850	0.650	0.550	0.750	0.650
π	0.0054	0.0067	0.0061	0.0069	0.0064	0.0044	0.0063	0.0034	0.0031	0.0012
π_a	0.0033	0.0040	0.0034	0.0043	0.0026	0.0032	0.0024	0.0021	0.0019	0.0006
π_s	0.0123	0.0151	0.0138	0.0154	0.0146	0.0099	0.0140	0.0076	0.0069	0.0032
Microsatellites										
$K_{average}$	12.8	7.8	8.5	7.1	7.3	8.3	8.6	8.9	7.0	8.5
K_{range}	8-20	4-12	7-13	4-9	4-10	4-11	5-15	5-12	4-12	6-12
H_E	0.811	0.768	0.785	0.750	0.727	0.816	0.797	0.778	0.745	0.791
H_O		0.713	0.763	0.756	0.700	0.794	0.769	0.769	0.694	0.763

B.	all	KN	KS	SI	MD	LL	HA	HS	IB	HE
N	127	17	18	6	16	18	11	15	17	9
TLR2										
h	5	1	2	3	2	2	3	4	2	2
h_{nons}	3	1	2	2	2	2	3	3	2	2
H_d	0.121	-	0.056	0.318	0.121	0.056	0.178	0.251	0.166	0.111
H_O		-	0.056	0.333	0.125	0.056	0.182	0.267	0.177	0.111
π	0.0002	0	0.0001	0.0003	0.0001	0.0001	0.0002	0.0002	0.0006	0.0004
π_a	0.0001	0	0.0001	0.0002	0.0001	0.0001	0.0002	0.0002	0.0002	0.0001
π_s	0.0004	0	0	0.0006	0	0	0	0.0002	0.0018	0.0012
Microsatellites										
$K_{average}$	11.8	6.6	6.4	5.0	6.0	6.0	6.0	7.0	5.8	5.2
K_{range}	4-22	2-10	2-9	2-8	2-9	2-10	3-9	3-12	2-9	2-8
H_E	0.702	0.614	0.652	0.639	0.679	0.589	0.694	0.756	0.666	0.749
H_O		0.565	0.633	0.700	0.688	0.633	0.691	0.733	0.588	0.733

Table 3. Estimation of pairwise genetic distance (F_{ST}) between bank vole (A) and yellow-necked mouse (B) populations based on microsatellite loci (below diagonal) and TLR2 (above diagonal). Bold values indicate that F_{ST} reached statistical significance after correction for multiple testing. KN, Kalvs mosse north; KS, Kalvs mosse south; SI, Silvåkra; MD, Myrdungen; LL, Lavelund; HA, Häglinge; HS, Hemmeströ; IB, Istaby; HE, Herseby.

A.	KN	KS	SI	MD	LL	HA	HS	IB	HE
KN	-	0.0149	0.0047	-0.0179	0.1364	-0.0214	0.1516	**0.1713**	**0.3538**
KS	**0.0227**	-	0.0007	-0.0018	0.0281	0.0149	0.0442	0.0583	**0.2117**
SI	**0.0460**	**0.0485**	-	-0.0093	0.0860	0.0029	0.1015	**0.1132**	**0.2984**
MD	**0.0406**	**0.0547**	**0.0534**	-	0.1080	-0.0186	0.1228	**0.1427**	**0.3252**
LL	**0.0499**	**0.0372**	**0.0372**	**0.0372**	-	0.1409	-0.0103	-0.0039	0.0807
HA	**0.0269**	0.0169	**0.0410**	**0.0401**	**0.0362**	-	**0.1570**	**0.1765**	**0.3674**
HS	**0.0430**	**0.0307**	**0.0407**	**0.0496**	**0.0502**	**0.0259**	-	-0.0198	0.1137
IB	**0.0800**	**0.0586**	**0.1249**	**0.1058**	**0.0883**	**0.0758**	**0.0787**	-	**0.1264**
HE	**0.0477**	**0.0338**	**0.0764**	**0.0669**	**0.0493**	**0.0388**	**0.0420**	**0.0787**	-

B.	KN	KS	SI	MD	LL	HA	HS	IB	HE
KN	-	-0.0016	0.0993	0.0351	-0.0016	0.0206	0.0225	0.0606	0.0377
KS	-0.0026	-	0.0260	0.0241	-0.0286	-0.0137	-0.0055	0.0463	0.0131
SI	0.0288	0.0270	-	0.0417	0.0260	-0.0201	-0.0466	-0.0042	-0.0278
MD	0.0235	**0.0405**	0.0171	-	0.0241	-0.0227	0.0016	0.0541	0.0250
LL	**0.0498**	**0.0361**	-0.0063	**0.0412**	-	-0.0137	-0.0055	0.0463	0.0131
HA	**0.0897**	**0.0615**	0.0212	**0.0666**	0.0488	-	-0.0330	0.0194	-0.0125
HS	**0.0551**	0.0423	0.0206	0.0366	**0.0693**	0.0239	-	0.0229	-0.0093
IB	**0.1137**	**0.0920**	**0.0857**	**0.0974**	**0.1052**	0.0251	**0.0503**	-	-0.0365
HE	**0.1225**	**0.0993**	**0.0902**	**0.1047**	**0.1156**	0.0460	0.0498	0.0312	-